ENRICHMENT OF DESEEDED CAROB POD WITH FUNGAL PROTEIN

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ABSTRACT - The carob tannin reduction and the enrichment of carob pod with fungal proteins by mixed cultures of *Aspergillus carbonarius* (Asca) and *Penicillium glabrum* (Pegl) in carob slurries, containing 2.5-15% carob and 0.5-2% $(NH_4)_2SO_4$, was studied. Maximum protein percentages (24-25%) were found in the cultures with 2.5-5% carob and 1.5% $(NH_4)_2SO_4$ concentrations, while the maximum production of total and fungal proteins were obtained in the media with 10-12.5% carob and 1.5% $(NH_4)_2$ SO_4. High tannin degradation was observed in low carob concentration (2.5-5%). The fermented carob pod, produced in 10% carob and 1.5% $(NH_4)_2SO_4$, was poor in nucleic acids (4.8%), ash (4.2%) and tannins (0.4%). In rat feeding trials, this fermented carob pod had the following nutritional indices: protein efficiency ratio (1.52 $\pm$ 0.20), net protein utilization (0.52 $\pm$ 0.01), biological value (0.62 $\pm$ 0.02) and true digestibility (0.72 $\pm$ 0.02).

RÉSUMÉ - Etude de la réduction en tannins des caroubes, ainsi que de l'enrichissement de la caroube en protéines fongiques, au moyen de cultures mixtes d'*Aspergillus carbonarius* (Asca) et de *Penicillium glabrum* (Pegl) dans un broyat de caroubes contenant 2,5-15% de caroubes et 0,5-2% de $(NH_4)_2SO_4$. Les meilleurs pourcentages de protéines (24-25%) sont obtenus dans les cultures contenant 2,5-5% de caroubes et 1-2% de $(NH_4)_2SO_4$ tandis que les productions maximales de protéines totales et de protéines fongiques sont atteintes dans les milieux contenant 10-12,5% de caroubes et 1,5% de $(NH_4)_2SO_4$. Les tannins sont mieux dégradés dans les cultures à basse concentration de caroubes (2,5-5%). Les caroubes fermentées, produites avec 10% de caroubes broyées et 1,5% de $NH_4)_2SO_4$ sont pauvres en acides nucléiques (4,8%), en cendres (4,2%) et en tannins (0,4%). L'estimation nutritionnelle de ces caroubes fermentées a été déterminée par des essais sur rats; les valeurs des indices de nutrition sont: le coefficient d'efficacité protéique (1,52 $\pm$ 0,20), l'utilisation protéique nette (0,50 $\pm$ 0,01), la valeur biologique (0,62 $\pm$ 0,01) et le taux réel de digestibilité (0,72 $\pm$ 0,02).

KEY WORDS : carob, carob beans, carob slurry, tannins, fungal proteins.

Source : MNHN, Paris

INTRODUCTION

The need for alternative protein sources has been universally recognised and extensive researches into new methods have been projected for increasing world protein production. This paper deals with one of these solutions, that is producing fungal protein biomass from carob bean (fruit of *Ceratonia siliqua* L. tree). This inexpensive substrate is a Greek agricultural product of low nutritional value.

The ripe carob pod (pericarp), although rich in water soluble sugar (about 60%) (Marakis, 1985a; Marakis & Karagouni, 1985) has a very low protein content (3-5%) (Marakis et al., 1987), and contains high levels of total tannins (6%) (Marakis & Karagouni, 1985) mainly condensed (Tamir & Alumot, 1970) which minimize the nutritional value of carob bean (Vohra et al., 1966; Tamir & Alumot, 1970). The grafted variety (g-3) carob pod contains 12.5% lignin, 4.8% hemicelluloses (Marakis, 1980) and 6% cellulose (Marakis et al., 1987).

Speculations on the carob bean value have been common during the last decade. The fungal protein production from aqueous carob extract was studied by several investigators (Buendia et al., 1961; Sekeri-Pataryas et al., 1973; Drouliscos et al., 1976; Macris & Kokke, 1977, 1978; Charpentié & Marakis, 1980; Marakis, 1980, 1985a; Marakis & Karagouni, 1985), but protein production from carob pod slurry has not been investigated yet. Marakis (1985b) carried out a preliminary research about the enrichment of the carob pod with fungal protein. In an experimental study of carob pod improvement, by solid-substrate fermentation, a cake-like structure product, containing ca. 7% protein, was produced (Kokke, 1977). The above carob enrichment is very low, because the unfermented carob pod contains about 5% crude protein. However, the carob pod direct enrichment with fungal protein had to be studied for two basic reasons: 1. The water soluble (sugars, tannins) and water insoluble (cellulose, lignin) carob components could be fermented. 2. The production-cost of the fungal protein will be lower, becouse no energy is needed for the preparation of aqueous carob extract, which is used in liquid cultures.

The problem that appears in this case, lies in the supply of a microorganism or microorganisms with a wide enzymatic system and able to consume more fundamental carob components (carbohydrates, lignin, tannins). For this purpose, we recently carried out a microbial screening, isolating fungi from the leaves of evergreen sclerophyllous shrubs (Maquis) (unpublished data).

This paper describes the enrichment of carob pod with fungal protein, as well as the chemical analysis and nutritional evaluation of the enriched fermentation product.

Source : MNHN, Paris

MATERIALS AND METHODS

Microorganisms

The carob pod is a "difficult" growth medium, as it was called by FAO/WHO/UNICEF Protein Advisory Group (Tate & Lyle, 1971), and the complementary culture system, a mixed culture of *Aspergillus carbonarius* (Asca) and *Penicillium glabrum* (Pegl), was used. These microorganisms, isolated from the leaves of evergreen sclerophyllous shrubs, were chosen, because, in preliminary experiments, presented a significant tannin-lignocellulolytic activity.

Growth media

A quantity of g-3 variety carob bean was broken in pieces 3-5mm long. 200g chopped and deseeded carob pod were boiled for 1 min with 700ml of distilled water. The infusion, after it had cooled, was stirred in a dough mixer for 2 min, and the resultant slurry made up to 1l. Na_2HPO_4 was added to give 0.1% slurry. This slurry was then diluted with $(NH_4)_2SO_4$ solutions, so that resultant media contained 0.5-2% $(NH_4)_2SO_4$ and 2.5-15% carob pod. The pH was buffered to 4.8-5. The carob slurry media were sterilized by autoclaving (15 min, 121°C).

Batch cultivation

Mixed cultures of *A. carbonarius* and *P. glabrum* were grown in 250ml Erlenmeyer flasks, containing 50ml of carob slurry. These flasks were inoculated with 2.10^7 spores of each used microorganism and incubated on reciprocal shaker (120 strokes per min) for 96h at 32°C. The above experiment was run in triplicate (3 flasks per run). The results were represented as mean values $\pm$ standard error.

Harvesting and drying of fermented carob pod (FCP)

The FCP was harvested by filtration through Whatman n° 1 filter paper, washed with 50ml of distilled water and dried by lyophilization to constant weight.

Analytical methods

- True protein determination (protein nitrogen X6.25) and extraction of nucleic acids were carried out by the Delaney et al. (1975) method. RNA and DNA were estimated by Gottlieb & Van Etten (1964) and Dische (1955) methods respectively, using baker's yeast RNA and calf thymus DNA (both Sigma Chemical Co. Ltd St. Louis U.S.A.) as standards.

- Total lipids were determined by Winter (1963) method.

Source : MNHN, Paris

- Amino acid analysis and determination of water-soluble tannins, ash, moisture and caloric content were carried out as Marakis (1985a) described.

- B-group vitamins were determined by Bell (1974) method.

- Cellulose and lignin were determined by the Jermyn & Isherwood (1956), Van Soest (1963), Kirk & Kelmen (1965) and Updegraff (1969) methods.

Nutritional evaluation

The nutritional indices measured were protein efficiency ratio (PER), biological value (BV), true digestibility (TD) and net protein utilization (NPU).

The procedure of the rat feeding and nutritional indices calculation, was carried out as Eggum (1973) and Malefaki-Perela (1981) described.

The composition of experimental diets was described by Marakis (1985a) with the difference that the 196g of *A. carbonarius* biomass were replaced by 305g of fermented carob pod (FCP).

RESULTS AND DISCUSSION

The effects of both ammonium sulfate and carob pod concentrations on dry weight and protein contents of FCP

The effects of these two substrate parameters on FCP dry weight and protein contents are shown in Table 1.

The higher percentage protein contents of the FCP occur in cultures containing lower carob pod concentrations. This may be due to the fact that it was impossible for the studied microorganisms to utilize equally, within 96h of incubation, the carob pod of the media on account of their different carob concentrations. As a result, the FCP proteins of the cultures, with higher carob concentrations, are distributed (diluted) into a greater amount of unutilized carob pod, in relation to the proteins of the lower carob concentrations. The latter (proteins), are distributed to a smaller amount of unconsumed carob pod. Thus, the lower the carob/$(NH_4)_2$ SO_4 ratio for any ammonium sulfate concentration the greater the protein content of the FCP was. This is in agreement with results reported by Marakis (1985b). The maximum (24-25%) protein contents of the FCP, occurring in the cultures with 2.5-5% carob and 1.5% $(NH_4)_2SO_4$ concentrations, are comparable to or higher than most of the other slurry single cell protein production systems (Chachal & Gray, 1969; Han, 1975; Peitersen, 1975; Brown & Fitzpatrick, 1976; Karapinar & Worgan, 1983), but lower than many of the liquid fermentations, which, of course, are solely the mycelial proteins. In primary liquid shaker mixed culture of *A. carbonarius* and *P. glabrum*, we found a

Source : MNHN, Paris

mycelium true protein content of 35%. It must be remembered that in liquid fermentations, there is no residual solid substrate to dilute the fungal protein, produced during fermentation.

Table I - Dry weight (mg/flask), percentage and quantity (mg/flask), of total protein, quantity of fungal protein (mg/flask) and yield (g of total protein/100g of carob pod) produced in carob pod slurries fermented by mixed culture of *A. carbonarius* and *P. glabrum*.

Tableau I - Poids de matière sèche, pourcentage et quantité de protéines totales, quantité de protéines fongiques et rendement produits dans les broyats de caroubes fermentées, au moyen de la culture mixte d'*A. carbonarius* et de *P. glabrum*.

Examined parameters	carob pod $(NH_4)_2SO_4$ (%)	2.5	5.0	7.5	10.0	12.5	15.0
FCP dry weight	0.5	779±4	1083±2	1498±5	2137±4	2570±7	2725±4
	1.0	778±3	1452±4	2075±3	2547±3	3319±3	3577±8
	1.5	782±2	1430±3	2293±2	3082±9	3985±5	3836±3
	2.0	796±5	1442±6	2180±4	2634±3	3564±6	3758±6
protein content/%	0.5	21.6	21.1	18.8	16.6	14.5	13.1
	1.0	23.3	22.3	21.3	19.4	17.5	15.1
	1.5	25.0	24.1	22.3	21.2	19.4	17.3
	2.0	22.2	21.2	20.5	19.8	17.1	15.2
total protein	0.5	168.3	228.5	281.6	354.7	372.7	357.0
	1.0	181.3	323.8	442.0	494.1	580.8	540.1
	1.5	195.5	344.6	511.3	653.4	773.1	663.6
	2.0	176.7	305.7	446.9	521.5	609.4	571.2
fungal protein	0.5	118.3	128.5	131.6	154.7	122.7	57.0
	1.0	131.3	223.8	292.0	294.1	330.8	240.1
	1.5	145.5	244.6	361.3	453.4	523.1	363.6
	2.0	126.7	205.7	296.9	321.5	359.4	271.2
yield	0.5	13.5	9.1	7.5	7.1	6.0	4.8
	1.0	14.5	13.0	11.8	9.9	9.3	7.2
	1.5	15.7	13.8	13.6	13.1	12.4	8.9
	2.0	14.1	12.2	11.9	10.4	9.8	7.6

The dry weight and the total protein (mg/flask) of the FCP increase with increasing the carob concentrations acquiring values up to 3985 and 773mg/flask respectively in cultures with 12.5% carob and 1.5% $(NH_4)_2 SO_4$. Production of protein could be obtained in concentrations of 773mg/flask, equivalent to 15.5g Total True Protein per Litre (TTPL), well in excess of other fungal slurry fermentations (Gray & Abou-el-Seoud, 1966a,b; Reade et al., 1972; Rogers et al., 1972; Marakis, 1985b).

Source : MNHN, Paris

By subtracting the protein content of the unfermented carob pod from the total protein FCP, the de novo protein amount due to fungal synthesis was calculated. The results reveal that the fungal protein production increased as carob pod concentration climbed up to 12.5%, acquiring maximum values (453-523mg/flask) in cultures with 10-12.5% carob and 1.5% $(NH_4)_2 SO_4$.

The relationship between the efficiency of protein production and carob pod as well as $(NH_4)_2SO_4$ concentrations showed a different figure, compared to the above mentioned results (see total and fungal proteins). Here, the yields (g of total protein produced per 100g carob pod) decreased as the carob concentrations increased and the $(NH_4)_2SO_4$ decreased. The maximum efficiency of protein production (15.7mg/100g carob) was observed in culture with 2.5% carob and 1.5% $(NH_4)_2SO_4$.

Having in mind the above data we can pose a question: which would be better for a single cell protein production process, a high TTPL or a high substrate conversion efficiency? If the cost of harvesting was of prime consideration then it might be advantageous to harvest a system producing maximal TTPL's. In this case, 10-15% carob with 1-2% $(NH_4)_2SO_4$ would be suitable. But, if maximum conversion efficiencies are sought, then 2.5-5% carob with 1-2% $(NH_4)_2SO_4$ would be more suitable. In the first case of fermentation, more power might be needed to aerate a 10-15% carob slurry than is necessary for cultures, containing 2.5-5% carob pod. The media with high carob concentrations should contain higher amounts of tannins and reducing sugars than the ones in media with low carob concentrations. But, high concentrations of readily consumed carob components (e.g. sugars) depress, at least in the first stages of the fermentation, the utilizing of other carbon sources (lignin, cellulose, etc.). Such a depression could have been caused by glucose presence (glucose effect). The poor aeration may also reduces the biomass yield (y = [g] mycelium dry weight/[g] consumed sugars). The significant fall of the fungal protein production in cultures with greater than the 12.5% carob concentrations is possibly due to high tannin and readily consumed sugar concentrations as well as to inadequate aeration. Therefore, the culture conditions, i.e. carob and $(NH_4)_2 SO_4$ concentrations, incubation time, as well as their effects on the tannin-lignocellulolytic activity have to be researched.

Tannin degradation

The percentage of tannin degradation, lightly increased with the increase of $(NH_4)_2 SO_4$, while a decrease was observed when carob concentrations increase, and particularly, in cultures with higher than the 10% carob pod (Tab. II). The maximum percentages (92-95%) of tannin degradation were observed in low carob concentrations (2.5-5%). *A. carbonarius* and *P. glabrum* under culture conditions, managed to degrade up to

Source : MNHN, Paris

240mg of tannins per flask, in the cultures containing 0.60-0.75% carob tannins (i.e. 10-12.5% carob pod).

Table II - Percentage of tannin degradation by mixed culture of *A. carbonarius* and *P. glabrum* in carob slurry media.

Tableau II - Pourcentage de tannins dégradés, au moyen de la culture mixte d' *A. carbonarius* et de *P. glabrum* dans des milieux de suspensions de caroubes.

Carob pod (%) $(NH_4)_2 SO_4$ (%)	2.5	5	7.5	10	12.5	15
0.5	92.3	92.1	86.0	75.2	57.1	41.2
1.0	93.4	93.2	86.8	78.8	61.0	48.0
1.5	94.8	94.5	89.8	80.8	64.1	51.1
2.0	95.4	95.2	90.4	81.7	66.2	52.6

The FCP produced in higher carob concentration cultures was richer in tannins than that in lower carob concentrations; From the total proteins/total tannins ratio in the FCP (Tab. III) we get the following results: The richer in proteins and poorer in tannins FCP was produced in cultures with lower carob and higher $(NH_4)_2 SO_4$ concentrations.

Table III - Total proteins/total tannins ratio of the FCP produced by mixed culture of *A. carbonarius* and *P. glabrum*.

Tableau III - Rapport des protéines totales sur tannins totaux de caroubes fermentées, au moyen de la culture mixte d'*A. carbonarius* et de *P. glabrum*.

Carob pod (%) $(NH_4)_2 SO_4$ (%)	2.5	5	7.5	10	12.5	15
0.5	69.4	44.7	35.1	31.5	25.7	22.3
1.0	87.1	74.6	66.3	51.1	45.6	39.7
1.5	103.7	88.1	80.9	70.3	65.4	50.6
2.0	106.0	87.0	79.2	67.3	60.3	48.1

The significant tannin reduction, constitutes a remarkable feature in the mixed culture of examined microorganisms, because carob pod is rich in condensed tannins which are resistant to microbial degradation. In aqueous carob extract fungal cultures (Sekeri-Pataryas et al., 1973; Macris & Kokke, 1977), the initial tannins did not affect the mycelial growth and not been consumed either. The tannin problem did not occupy the above workers, although tannins play an important role in the decrease of carob nutritional value.

Kokke (1977) found that fermented products in carob solid-fermentations gave negative values of tannins. It seems unlikely, because, as he reported, water soluble sugars were not completely used. He rather deter-

Source : MNHN, Paris

mined a tannin fraction (e.g. tannic acid) than the total tannin contents of the fermented carob pod.

Gross composition of the FCP

Table IV presents the gross composition and metabolizable energy of the FCP produced in culture with 10% carob and 1.5% $(NH_4)_2$ SO_4. This FCP was chosen for chemical analysis and nutritional evaluation due to the maximum amounts (mg/flask) of tannin and lignocellulose degradation, and the culture aeration, in 10% carob, being easier than that in cultures with higher carob concentrations; although in the latter, 16% higher de novo protein was produced. The true protein content (21%) of the FCP is higher than that (7%) of crude protein reported by Kokke (1977). The FCP protein and ash contents are considered to be acceptable. From a nutritional aspect it is important that ash percentage is low, normally less than 5% in the compounded feed. The nucleic acid and tannin contents of the FCP are low. Tannin level (0.4%) has been shown not to detract the nutritional quality of FCP.

Table IV - Gross composition and metabolizable energy of the FCP produced by mixed culture of *A. carbonarius* and *P. glabrum* in medium with 10% carob and 1.5% $(NH_4)_2$ SO_4. (239 Kcal = 1 MJ of metabolizable energy).

Tableau IV - Composition et énergie métabolisable de caroubes fermentées au moyen de la culture mixte d'*A. carbonarius* et de *P. glabrum* dans un milieu à 10% de caroubes et à 1,5% de $(NH_4)_2$ SO_4. (239 Kcal = 1 MJ de l'énergie métabolisable).

True protein	21.2% on dry weight
Total nucleic acids	4.8% on dry weight
Total tannins	0.4% on dry weight
Total lipid	6.1% on dry weight
Cellulose	4.5% on dry weight
Lignin	9.1% on dry weight
Ash	4.2% on dry weight
Moisture	4.7% on dry weight
Pyridoxine	119.0 μg/g of dry weight
Biotin	1.1 μg/g of dry weight
Pantothenic acid	39.0 μg/g of dry weight
Nicotinic acid	108.5 μg/g of dry weight
B_2	68.1 μg/g of dry weight
B_{12}	23.2 μg/g of dry weight
Metabolizable energy	10.8 MJ/Kg of dry weight

The deseeded carob pod contains 12.5% lignin and 6% cellulose (Marakis, 1980; Marakis et al., 1987), while the cellulose and lignin contents of FCP was 4.5% and 9.1% respectively. On the basis of the above contents and given that the FCP dry weight was 3.1g/flask, the cellulose and lignin degradation was found to be 53% and 55% (on the initial content) respectively. For the utilization of cellulose and hemicelluloses it is necessary to

Source : MNHN, Paris

degrade lignin (Asther et al., 1987). Since cellulose and lignin are of little nutritional value, because they affect the true nitrogen digestibility (Malefaki-Perela, 1981), these substances should be even more degraded. This might probably be achieved by prolonging the incubation time beyond 96h. The role of tannins on the decomposition of cellulose, hemicelluloses and lignin by fungal activity was not discussed here. In preliminary experiments an intense cellulase and ligninase activity was found in mixed cultures of *A. carbonarius* and *P. glabrum*. Considerable effects, in this field, are taking place in our laboratory nowadays.

B-group vitamin contents of the FCP, satisfy the requirements for a microbial protein production system. The riboflavine content is in agreement with that reported by Marakis (1985a), but it is 70% higher than the observed by Forage (1978). Metabolizable energy is at a level comparable to that reported by Marakis (1985a), Forage (1978) and Sell et al. (1981).

The amino acid composition of FCP (Tab. V) is favourably compared with the Smith et al. (1975) references for the growing rat and pig requirements and the United Nations FAO/WHO (1965) reference protein; except sulfur-amino acid contents which were low, like many microbial proteins. Total essential amino acid content was comparable or superior to other microbial products (Drouliscos et al., 1976; Kokke, 1977; Marakis, 1985a) and certain agricultural by-products of vegetable origin (Malefaki-Perela, 1981).

Table V - Amino acid composition (g/16g N) of the FCP produced by mixed culture of *A. carbonarius* and *P. glabrum* in medium with 10% carob and 1.5% $(NH_4)_2 SO_4$.

Tableau V - Composition en acides aminés (g/16 g N) de caroubes fermentées, au moyen de la culture mixte d' *A. carbonarius* et de *P. glabrum* dans un milieu à 10% de caroubes et à 1,5% de $(NH_4)_2 SO_4$.

Phe	5.1	Arg	5.2
Tyr	4.5	Trp	0.7
His	3.1	Total EAA	51.0
Ile	5.4	Asp	9.2
Leu	6.8	Ser	4.1
Lys	5.4	Glu	12.5
Met	1.7	Pro	3.6
Cys	1.6	Gly	3.9
Thr	5.0	Ala	5.5
Val	6.5	Total AA	89.8

Nutritional quality of the FCP

An examination of the Table VI shows that PER and NPU values of FCP are lower than those of soya bean oil meal, but higher than the ones found by Smith et al. (1975) and Drouliscos et al. (1976). The TD is generally low. This could be due to a certain lignocellulose content of the FCP.

Source : MNHN, Paris

Malefaki-Perela (1981) found a negative correlation between true digestibility and lignocellulose. Comparison of our data with previously reported nutritional indices of *A. carbonarius* (AsDT10) biomass, grown in aqueous carob extract (Marakis, 1985a), showed that the FCP was of a lower nutritional quality than the one of *A. carbonarius* biomass. In our case, the compound, cellulose or lignin, affecting more the nutritional indices is unknown. This problem is open for discussion. Tannin content (0.4%) of the FCP does not appear to present a toxicological problem or to depress protein digestibility and utilization. During the experimental period, rats didn't lose their appetite.

Table VI - Nutritional indices of FCP produced by mixed culture of *A. carbonarius* and *P. glabrum* in medium with 10% carob and 1.5% $(NH_4)_2 SO_4$.

Tableau VI - Indices de nutrition de caroubes fermentées, au moyen de la culture mixte d'*A. carbonarius* et de *P. glabrum* dans un milieu à 10% de caroubes et à 1.5% de $(NH_4)_2 SO_4$.

Indices	Diets	
	Fermented residue	Soy bean oil meal
Protein efficiency ratio (PER)	1.52±0.20	2.6±0.15
Biological value (BV)	0.62±0.01	0.68± 0.02
True digestibility (TD)	0.72±0.02	0.89±0.01
Net protein utilization (NPU)	0.52±0.01	0.60±0.01

Which protein production system is better? one or two-step carob pod fermentations?

In reply to the above question and in order to establish an econometric model for the upgrading of carob value, the growth of mixed culture of *A. carbonarius* and *P. glabrum* should be separately studied in aqueous carob extract and spent carob (extracted carob pod).

We supposed that microorganisms must use the carob components (carbohydrates, lignin, tannins, etc.) differently, when they are all together (one-step) than when they are found separately (two-step system). This happens because the presence of one component in the substrate influences the utilization of the other (e.g. glucose effect phenomenon).

Marakis (1980) in a preliminary study about fungal protein production by fermenting carob pod in the two-step system, observed that the total protein (mg/flask) was higher in two-step system fermentation than those in one-step system. While protein productivity (g/l of medium/h) was 20% higher in one step than that in two-step fermentation system.

Source : MNHN, Paris

CONCLUSION

The mixed culture of *A. carbonarius* and *P. glabrum* in carob pod slurries gave a fermented product relatively rich in true protein with balanced amino acid profile, poor in nucleic acids, ash and tannins. Although, the degradation of cellulose and lignin was moderate, the results obtained from this study suggest that the above mixed culture could be considered a safe and promising system, to use for improving carob bean value.

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Source : MNHN, Paris

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